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TECHNICAL MANUSCRIPT 414

PATHOLOGICAL CHANGES IN RABBITS
INJECTED WITH PASTEURELLA TULARENSIS
KILLED BY IONIZING RADIATION

Milton J. Finegold
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DECEMBER 1967

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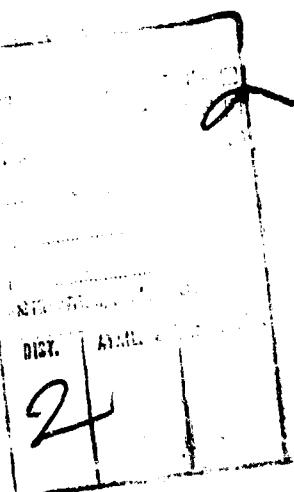
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DEPARTMENT OF THE ARMY
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TECHNICAL MANUSCRIPT 414

**PATHOLOGICAL CHANGES IN RABBITS INJECTED WITH
PASTEURELLA TULARENSIS KILLED BY IONIZING RADIATION**

Milton J. Finegold

James D. Pulliam

Marshall E. Landay

George G. Wright

Pathology Division
and
Medical Investigation Division
MEDICAL SCIENCES LABORATORY

Project 1C522301A059

December 1967

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

Suspensions of Pasteurella tularensis killed by exposure to ionizing radiation were toxic for rabbits. A dose of 3 ml or more of a suspension containing 2×10^{11} to 3×10^{11} organisms per ml was lethal within 24 hours of a single intravenous injection. The major pathologic changes were extensive hemorrhagic necrosis of the spleen, focal coagulation necrosis of the liver, pneumonitis, and glomerular capillary occlusion by fibrin thrombi. Similar lesions were produced when two smaller doses of the suspension were given intravenously 24 hours apart. The localized Shwartzman reaction was produced by an intradermal injection followed in 24 hours by an intravenous injection. Rabbits were protected against the lethal action of the suspension and against development of glomerular thrombosis by prior administration of either a single dose or of multiple doses of 25 mg of cortisone.

The pathological findings were similar to those in rabbits given endotoxins from meningococci or other gram-negative organisms and may be interpreted as a combination of the local and generalized Shwartzman reactions. Similarities were also noted between the responses to killed P. tularensis and those occurring during experimental tularemia infection in rabbits.

I. INTRODUCTION

Suspensions of Pasteurella tularensis killed by exposure to ionizing radiation retain a significant toxicity for mice, guinea pigs, and rabbits. Responses to injection of the suspension were complex, resembling those typical of endotoxin in some respects but not in all.* In rabbits, the endotoxin-like manifestations appeared to predominate, as shown by fever and the protection afforded by cortisone. Injection of newly prepared suspensions into mice revealed a toxicity against which cortisone was ineffective, although tolerance was induced by prior injection of endotoxin of Escherichia coli. This toxicity deteriorated as the suspensions stood at 4 C, leaving an endotoxin-like activity against which mice could then be protected by cortisone. In an effort to understand more fully the responses to injection of the irradiated suspensions, and to determine their relationship to changes associated with exposure to viable organisms,^{1,2} studies were carried out on tissue changes in rabbits injected with the irradiated suspensions.

II. MATERIALS AND METHODS

Bacterial suspensions were prepared as described elsewhere* that contained 2×10^{11} to 3×10^{11} viable organisms per ml prior to receiving 1×10^6 r of gamma radiation from a high-level Co⁶⁰ source. Sterility of the suspension was established by inoculation onto SB agar medium.³ Dilutions of the suspension, when required, were prepared in pyrogen-free 0.9% sodium chloride solution.

New Zealand white rabbits of both sexes were used as young adults, weighing from 1.5 to 2.5 kg. They were fed Purina rabbit checkers and allowed water ad lib. Injections were made into a marginal ear vein. Routine necropsies were performed on animals after death or after sacrifice with carbon dioxide or intravenous pentobarbital. Sections of lung, spleen, liver, kidney, and sometimes heart were fixed in 10% neutral formalin, embedded in paraffin, and sectioned at 5 to 7 μ . Sections were stained with hematoxylin and eosin, phosphotungstic acid - hematoxylin, alcian blue - periodic acid Schiff, or alizarin red, as required. Representative pathological findings are shown in Figures 1-6.

* Landay, M.E., G.G. Wright, J.D. Pulliam, and M.J. Finegold. 1967. Toxicity of Pasteurella tularensis killed by ionizing radiation. Unpublished data.

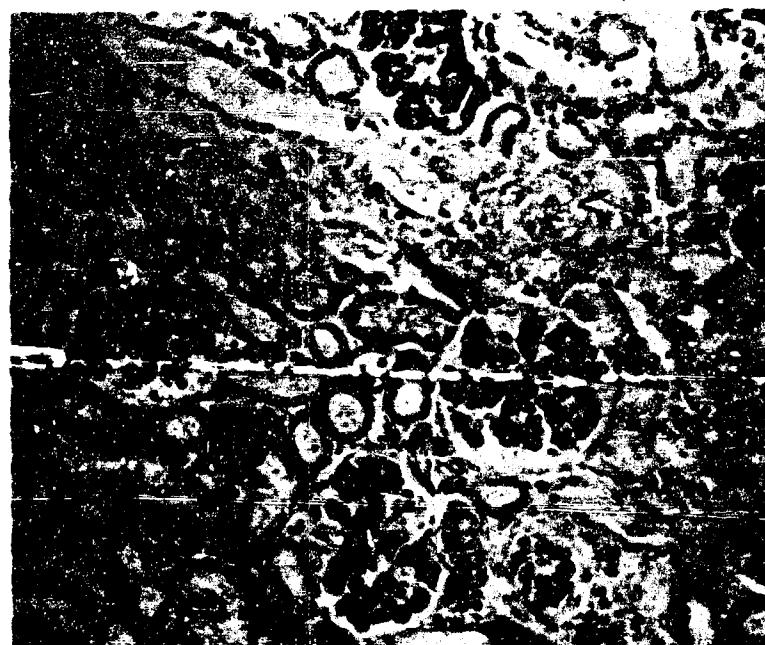


Figure 1. Kidney. Glomerular capillaries are occluded by fibrin thrombi. Phosphotungstic acid - hematoxylin. 130X.



Figure 2. Spleen. Massive necrosis is manifest by nuclear debris and disruption of the architecture. Hematoxylin and eosin. 130X.

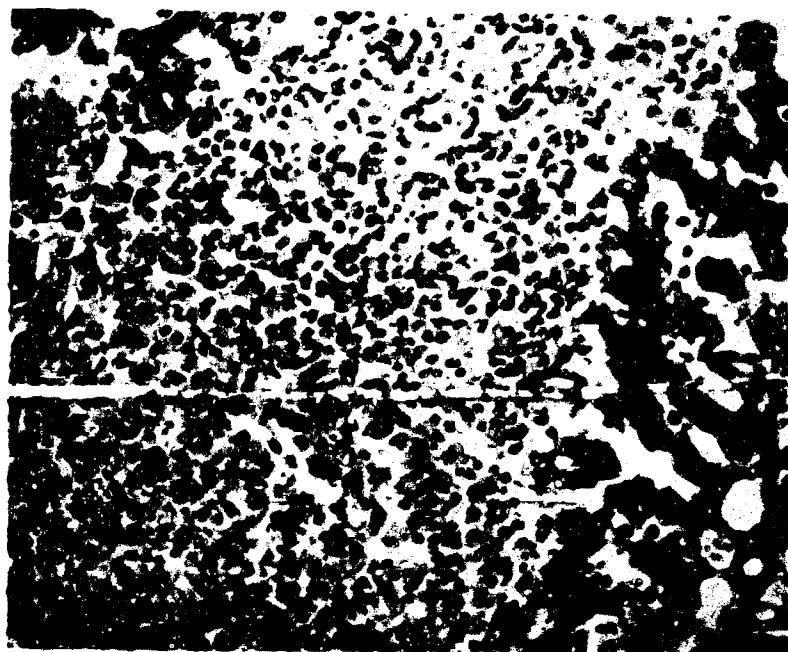


Figure 3. Liver. Coagulation necrosis of virtually an entire lobule is shown. The central vein is at upper left and the portal area is to the lower right. Hematoxylin and eosin. 420X.



Figure 4. Lung. Pulmonary arteritis is characterized by fibrinoid necrosis, polymorphonuclear leukocyte infiltration of the intima and inner media, and swelling of remaining endothelial cells. There is interstitial hemorrhage in the adjacent tissue. Hematoxylin and eosin. 42X.



Figure 5. Heart. Calcification of myocardial fibers is shown by the extensive blackening of the mineralized muscle. Alizarin red. 130X.

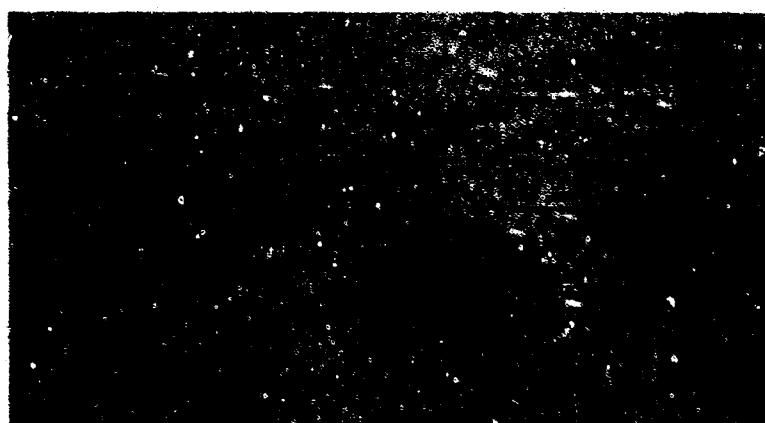


Figure 6. Localized Shwartzman Reaction. Each of the three sites of hemorrhagic necrosis received an intradermal injection of irradiated *P. vulgaris* suspension 24 hours prior to intravenous injection of the same material. These areas of necrosis were photographed 18 hours after the intravenous injection.

III. RESULTS

A. SINGLE INJECTIONS

Twenty-one rabbits received from 0.125 to 9 ml of suspension in a single intravenous injection. The earliest deaths occurred at 11 hours, with doses of 3, 6, or 9 ml (Table 1). Nine of the 10 rabbits given 3 ml or more of suspension died within 21 hours; the other was sacrificed when moribund at 20 hours. The spleen, examined in nine animals, was diffusely necrotic and hemorrhagic in eight, with lymphoid cell depletion and hemorrhage in the other animal. The liver of six rabbits showed a variable degree of focal hepatocellular necrosis, ranging from individual cell hyalinization to 3-mm areas of coagulation necrosis with a granulocytic exudate. Pneumonitis was found in eight of the nine pairs of lungs examined; it was characterized by a diffuse septal, perivascular, and peribronchial polymorphonuclear leukocytic infiltrate. Also present were a variable increase in mononuclear cells in the septa, focal interstitial and alveolar edema, and small hemorrhages. The kidneys of seven of these 10 rabbits displayed occlusion of glomerular capillaries by fibrin thrombi (GFT) to a variable degree, ranging from an occasional thrombosed loop to complete occlusion. Full-blown gross cortical necrosis was not observed, but swollen, pale cortices were seen in those kidneys with microscopic necrosis due to extensive thrombosis. The proximal tubular epithelium in the non-necrotic areas of these seven kidneys frequently contained hyaline droplets or clear vacuoles, and colloid casts were numerous. The incidence of these changes is summarized in Table 1.

Of 11 rabbits given less than 3 ml of suspension, two died after 20 and one after 26 hours. The three had received 1 ml and had extensive splenic necrosis, focal hepatic necrosis, and pneumonitis. Two of the three had GFT, which were of sufficient extent in one case to produce cortical necrosis. None of the eight animals sacrificed between 23 and 85 hours after injection had GFT, and there were fewer and smaller lesions in the other organs.

B. MULTIPLE INJECTIONS

Detection of GFT in nine of 12 rabbits dying after a single injection of irradiated suspension indicated the occurrence of disseminated intravascular coagulation,⁴ a finding compatible with the presence of endotoxin-like activity. Accordingly, the capability of the suspension to produce the generalized Shwartzman reaction was investigated.⁵

TABLE 1. RESPONSES OF RABBITS TO INTRAVENOUS INJECTION OF
IRRADIATED *PASTEURELLA TULARENSIS* SUSPENSION

Schedule, Dose, Other Treatment	No. of Rabbits	Deaths	Interval from Last Dose to Death, hours	Incidence of Lesions ^a			
				Focal Liver	Glomerular Pancreatitis	Splenic Necrosis	Glomerular Vasculitis Necrosis
Single dose							
<3 ml	11	3	20 - 26	5	8	4	2
3 to 9 ml	10	9	11 - 21	8 of 9	6	8 of 9	7
Multiple doses, 24 hours apart							
0.1 or 0.2; 2 ml	11	6	12 - 96	7 of 8	7 of 8	3 of 8	7
0.1 ml; 2 ml; 2 ml	6	0	-	4	2	4	1
Cortisone, 12.5 ug 3 ml suspension	10	0	-	7	8	8	1
Prior splenectomy							
1 or 3 ml dose	6	5	10 - 21	-	6	6	3
0.2 and 2 ml doses	3	3	19 - 22	-	3	3	0

a. All animals in the group were examined, except where a smaller number is indicated.

Sublethal preparative doses of 0.1 or 0.2 ml were given intravenously to 17 rabbits. A provocative dose of 2 ml was given 24 hours later. Three rabbits died within 12 to 20 hours after the second injection, and all had GFT. Five rabbits were sacrificed between 19 and 24 hours after the second injection; GFT were present in three. Three rabbits died after 48, 54, and 96 hours, respectively. Only the 48-hour animal had GFT but all three had extensive thrombi in pulmonary arteries, arterioles, or capillaries; pulmonary infarction was present in the 96-hour specimen. The thrombi in the lungs sometimes differed from those in the kidney in having leukocytes and erythrocytes enmeshed in the fibrin. Some pulmonary arteries and arterioles displayed various degrees of fibrinoid necrosis, endarteritis, intimal fibrosis, and calcification of the fibrous tissue. Frequently, arteries that were neither thrombosed nor inflamed showed medial muscular thickening. These changes are similar to those described after a single injection of meningococcal endotoxin in rabbits⁶ and those seen in rabbits given live meningococci intradermally followed by live meningococci intravenously 24 hours later.⁷

Six of the nine rabbits that survived 24 hours after the provocative injection were given an additional 2 ml of suspension intravenously between 27 and 30 hours after the second injection. Only the rabbit sacrificed 8 hours later (35 hours after the second injection) had GFT, but it and the two animals sacrificed 20 and 23 hours after the third dose had pulmonary arterial thromboses with vasculitis and infarction. A pulmonary infarct was also found in one of the three rabbits that were sacrificed 69 hours after the third injection.

In summary, eight of ten rabbits necropsied within 48 hours of the provocative injection, including one that received a third dose of the suspension 8 hours before sacrifice, had GFT. Of nine rabbits that survived 24 hours or more after the provocative injection, seven had pulmonary thrombi, including four that had received a third dose of the suspension. Two rabbits that died between 24 and 48 hours had fibrin thrombi in both kidneys and lungs.

C. INTRADERMAL INJECTION

The local Shwartzman reaction⁸ was produced in rabbits by intradermal injection of 0.1 ml volumes of serial dilutions of irradiated suspension from undiluted to 1:2048, followed 24 hours later by 0.5 ml of suspension injected intravenously. Positive reactions developed within 18 hours at all dilutions tested. They consisted of areas of induration 2 to 2.5 cm in diameter with central hemorrhagic necrosis. The preparatory injections alone caused slight swelling at the sites of injection after 24 hours.

D. CORTISONE TREATMENT

The protective effect of cortisone against the lethal action of the suspension in rabbits was studied further in an effort to determine which lesions were inhibited by the drug. Ten rabbits were injected subcutaneously with 12.5 mg cortisone acetate, followed immediately by 3 ml of suspension injected intravenously. This dose of suspension alone killed six of seven untreated rabbits within 18 hours; the seventh was moribund and was sacrificed at 12 hours. GFT were present in four of the seven. None of the cortisone-treated animals died, so they were sacrificed in pairs 25, 45, 78, 98, and 121 hours after injection. GFT were found in only one rabbit, an animal sacrificed at 25 hours. Pulmonary vasculitis was present in seven of eight rabbits examined after 45 hours or more. Splenic necrosis was extensive in seven animals. Focal hepatic necrosis was present in eight, with dystrophic calcification of such foci after 45 hours or more. The myocardium of several animals contained foci of coagulation necrosis with dystrophic calcification. Coronary vascular lesions were sought, with negative results; they had been observed in rabbits injected once with meningococcal endotoxin.⁵ Thus, a single injection of cortisone acetate, which protected 10 of 10 rabbits against a lethal dose of the suspension, did not alter the pathologic responses of the spleen, liver, and lung.

In contrast to the protective action of a single treatment with cortisone,⁹ multiple injections of cortisone in rabbits had been shown to potentiate the lethality and the Shwartzman reactivity of a single injection of endotoxin.⁶ The effect of cortisone administered in a similar manner was tested with the P. tularensis suspension. Twelve rabbits weighing 1 kg each were treated intramuscularly with three daily doses of 25 mg cortisone acetate prior to a single intravenous injection of either 1 ml (six rabbits) or 0.5 ml (six rabbits) of suspension. Controls of the same weight were given 1 ml (six rabbits), 0.5 ml (six rabbits), or 0.25 ml (four rabbits) of suspension.

All controls developed diarrhea, hematuria, and lassitude; the cortisone-treated animals remained clinically well. Two controls died 16 and 19 hours after receiving 1 ml of suspension. All other animals were sacrificed and necropsied at 24 hours. Fourteen of 16 controls had GFT, with gross cortical necrosis in five. The two negative rabbits had received 0.25 ml of suspension. None of the 12 cortisone-treated rabbits had GFT, although they had necrotic lesions in the spleen and lungs that were similar to those in the controls. Thus, cortisone administered in either single or multiple injections protected against both the Shwartzman reaction and lethal action of P. tularensis suspension.

E. SPLENECTOMY

The occurrence of extensive hemorrhagic necrosis of the spleen in 17 of 18 rabbits that died, and the presence of GFT in 13, suggested that intravascular coagulation might have been initiated by tissue thromboplastins⁴ released into the circulation from the necrotic spleen. Therefore, the effects of splenectomy on responses to the suspension were investigated. Nine rabbits, splenectomized 25 or 26 days previously, were injected intravenously with 1 ml or 3 ml doses, or with 0.2 and 2.0 ml doses 24 hours apart (Table 1). Eight animals died within 44 hours; the survivor was sacrificed after 54 hours. All showed focal hepatic necrosis and pneumonitis. Pulmonary arteries and arterioles contained fibrin thrombi in five of the nine, and GFT were found in three. Prior removal of the spleen evidently had no effect on pathological reactions to the suspension.

IV. DISCUSSION

The pathological responses of rabbits to intravenously administered suspensions of radiation-killed *P. tularensis* are similar to those of rabbits dying acutely of experimental tularemia infection.¹ In both situations, splenic and hepatic necrosis and pulmonary and glomerular capillary occlusion by fibrin thrombi are common findings. This suggests that the toxicity of the irradiated suspension may be related to pathogenic mechanisms of infection with live organisms and justifies attempts to determine more precisely the nature of the toxicity.

Rabbits given a single large intravenous dose of meningococcal endotoxin⁶ or two injections of live washed meningococci, the first intradermally and the second intravenously 24 hours later,⁷ develop visceral lesions comparable to those produced by the *P. tularensis* suspension. Both the necrotic manifestations and intravascular coagulation leading to glomerular capillary occlusion are features of the generalized Shwartzman reaction, with fibrin thrombi considered to be characteristic of the reaction.^{4,8} The sequential changes in the lungs, with diffuse granulocytic infiltration in the early stages, followed by thromboses and vasculitis with frequent infarction, are typical of the local Shwartzman phenomenon.⁹ Classically, both the generalized and localized phenomena are produced by two injections of gram-negative endotoxin spaced 24 hours apart,⁸ but the local reaction has been produced by a large single dose of endotoxin,¹⁰ and a number of techniques not involving endotoxin have been used to duplicate the essential findings of both reactions.⁴ It would therefore be inappropriate to conclude that the toxic principle is endotoxin merely because the responses of rabbits to irradiated *P. tularensis* closely resemble the Shwartzman reaction.

The only evidence of the participation of endotoxin in tularemia, other than the suggestive pathological responses in rabbits,¹ is the demonstration that volunteers convalescing from experimental tularemia infection became tolerant to the pyrogenic action of Salmonella typhosa endotoxin in the same manner as patients with typhoid fever.¹¹ Brief reference was also made to unpublished reports of isolation of material from P. tularensis with properties of endotoxin, including pyrogenicity, lethality for rabbits, and the ability to induce the Shwartzman reaction. However, the lipopolysaccharide extracted from P. tularensis by Stefanye¹² was neither pyrogenic nor lethal for mice in amounts up to 1.0 to 2.0 mg. To date, definitive chemical isolation of endotoxin from P. tularensis has not been reported.

The protective activity of cortisone tends to support the concept that endotoxin activity contributes significantly to the lethality of the irradiated suspension for rabbits. The results are similar to those obtained with a single injection of cortisone prior to treatment with meningococcal endotoxin.⁹ However, results with multiple injections of cortisone were different in the two systems; this treatment potentiated the classical endotoxin⁵ but protected against the P. tularensis suspension.

The association between the protective effect of cortisone and the prevention of fibrin thrombi in the glomerular capillaries of 21 of the 22 rabbits so treated implies that intravascular coagulation is responsible for early deaths. The incidence of visceral necrosis is the same in both treated and untreated animals that survived for the same period. However, glomerular thrombi were not found in four of 14 control rabbits without cortisone that died within 24 hours of injection, so a crucial role for intravascular coagulation in the lethality of the suspension appears questionable. Evidently, intravascular coagulation is one of several manifestations of toxemia, rather than the primary cause of death in this toxemia or in tularemia; alterations in cellular metabolism and physiology may be the crucial pathogenetic mechanisms.^{13,14}

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Unclassified

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)		2a. REPORT SECURITY CLASSIFICATION Unclassified	2b. GROUP
Department of the Army Fort Detrick, Frederick, Maryland, 21701			
3. REPORT TITLE PATHOLOGICAL CHANGES IN RABBITS INJECTED WITH <u>PASTEURELLA TULARENSIS</u> KILLED BY IONIZING RADIATION			
4. DESCRIPTIVE NOTES (Type of report and inclusive dates)			
5. AUTHOR(S) (First name, middle initial, last name)		Marshall E. Landay George G. Wright	
6. REPORT DATE December 1967		7a. TOTAL NO. OF PAGES 16	7b. NO. OF REPS 14
8. CONTRACT OR GRANT NO.		9a. ORIGINATOR'S REPORT NUMBER(S)	
a. PROJECT NO. 1C522301A059		Technical Manuscript 414	
a.		9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
a.		a.	
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11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY Department of the Army Fort Detrick, Frederick, Maryland, 21701	
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14. Key Words * <u>Pasteurella tularensis</u> *Pathology Rabbits Radiation Vaccines			

DD FORM 1473

Unclassified

Security Classification